SYNOPSIS

Title of the Ph.D thesis: Modulatory Effect of Glycosaminoglycans (GAGs) from Fish Waste on Obesity Induced Atherosclerosis

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There has been a sustained growth in the supply of fish from the last 50 years with an average growth rate of 3.2% globally [Ghaly et al., 2013]. India is the second largest producer of fish in the world [FAO, 2018]. Fish processing industry uses 70% fish for fillets and 30% is discarded as a non-edible by-product in the form of fins, frames, heads, and skin, which create environmental pollution [Islam et al., 2004]. Indian fish export reached 11.5 lakhs in 2015- 2016 of which roughly 50% i.e. 5.5 lakhs tonnes has been disposed as wastes [Ahmad & Bhuimbar, 2019]. Global fish waste generation is estimated to be in excess of 100 mMT, and in the Indian scenario it is >4 mMT [Binsi, 2021].

Around 60% of the fish that are caught globally are either processed into frozen, canned, or preserved foods or used in fresh fish markets, which generates a lots of waste materials. Presently, the majority of these waste materials are disposed into landfills or transformed on a small scale into goods like animal feed and leather, these waste poses considerable environmental risks. During processing, the amount of waste generated ranges from 20-80%, depending on the level of processing and type of fish [Ghaly et al., 2013], these wastes can be utilized, as they are a good source for recoverable macro and micronutrients. An effective waste management strategy generally involves waste minimization, waste characterization, and waste utilization [Ahmad & Bhuimbar, 2019].

With rapid developments in the field of biotechnology, there are numerous opportunities to use these wastes as sources of recoverable nutraceuticals and other valuable functional molecules, which include proteins like collagen, gelatin, protein hydrolyzates, bioactive peptides, lipids rich in polyunsaturated fatty acids, squalene, polysaccharides like chitin, chitosan, glycosaminoglycans *etc*., [Brito et al., 2008; Babu et al., 2008]. Researchers across the globe are focusing on methods to recover the valuable biomolecules in order to reduce organic loading to the environment and decrease the pollution related problems.

Obesity prevalence has significantly increased in recent years and is now a major global public health issue. It has more than doubled globally between 1980 and 2014 [WHO, 2017]. It is characterised with a body mass index, BMI > 30 kg/m^2, and an BMI: 25-30 kg/m^2 is considered overweight [Beltowski, 2006]. Around 30% of the world's population, or two billion people over the age of 18, are overweight or obese [Ng et al., 2014]. Obesity is now recognized as only one among many components of the metabolic syndrome, according to the International Diabetes Federation [IDF, 2005]. A strong association has been established between obesity and chronic diseases, such as diabetes, hypertension, hyperlipidemia, cardiovascular disease, cancer, and other metabolic syndromes [Grundy et al., 2004,

Swinburn et al., 2011]. Obesity results from energy imbalance, which is influenced by lifestyle, high-fat diet, and other behavioural, environmental, and genetic factors. Some of the major obesity characteristics are dysregulation in lipid metabolism and insulin resistance [Pasquinelli, 2012]. By 2045, 51% of the global population are estimated to be obese [Saeedi et al., 2019].

Obesity is a significant contributor to the risk of developing a number of diseases, such as type 2 diabetes, non-alcoholic fatty liver disease, hypertension, and cardiovascular disease (CVD) [Velasquez-Mieyer et al., 2005]. It is now widely recognised that chronic low-grade inflammation plays an important role in the initiation, propagation and development of metabolic diseases [Baker et al., 2011]. Being obese or overweight poses a significant risk factor for atherosclerosis [Poirier & Eckel, 2002]. Adipose tissue regulates the production of hormones, angiogenic factors, and cytokines, adipocytes can secrete pro-angiogenic molecules, as a risk factor for atherosclerosis [Ahmadieh et al., 2020].

Atherosclerosis is a chronic disease characterized by lipid retention and inflammation in the artery wall. The retention and oxidation of low-density lipoprotein (LDL) in sub-endothelial space play a critical role in atherosclerotic plaque formation and destabilization [Tian et al., 2020]. Majorly oxidized low-density lipoproteins (ox-LDL) play an important role in atherosclerotic plaque formation [Kataoka et al., 1999]. Atherosclerosis, is mainly caused by dyslipidemia and characterized by the accumulation of macrophages and products of lipid and protein oxidation in blood vessels, is a fundamental pathological process for the development of cardiovascular disease [Rong et al., 2018]. Lipid metabolism disorder, oxidative stress, chronic inflammation and endothelial dysfunction play key roles in the development and progression of atherosclerosis [Poznyak et al., 2020].

Despite the enormous progress made in terms of its prevention and early intervention, a pressing need remains to develop innovative therapeutic strategies for ischemic cardiovascular disorders, including acute myocardial infarction, chronic cardiac ischemia, peripheral artery disease and stroke.

Polysaccharides are already being widely used in various industries, also they are most exploited molecules, it has been noted that there is a gradual rise in their application in more advanced therapeutic formulations. Glycosaminoglycans (GAGs) is one of the crucial bioactive polysaccharides, which is well documented as a health beneficial molecule [Reynés et al., 2016; Nogueira et al., 2019; Wang et al., 2017]

In recent years, GAGs isolated from natural resources have attracted much attention due to their wide variety of pharmacological activities. These are long, unbranched heteropolysaccharides which are found in an extensive array of organisms and execute many physiological and biological roles. GAGs are linear, negatively charged, either sulphated or non-sulphated, polysaccharides that have molecular weights of roughly 10-100 kDa. There are two main types of GAGs. Non-sulphated GAGs include hyaluronic acid (HA), whereas sulphated GAGs include chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparin (HEP) and heparan sulphate (HS) [Gandhi & Mancera, 2008].

GAGs chains are composed of disaccharide repeating units, the repeating units are composed of uronic acid (D-glucoronic acid or L-iduronic acid) and amino sugar (D-galactosamine or D-glucosamine). Chondroitin sulphate and dermatan sulphate, which contain galactosamine, are called galactosaminoglycans, whereas heparin and heparan sulphate, which contain glucosamine, are called glucosaminoglycans [Gandhi & Mancera, 2008].

GAGs interact with a variety of proteins, including growth factors, enzymes, and cytokines, thereby play a part in a number of pathophysiological processes. It is also involved in lipid transport and metabolism, cell-to-matrix and cell-to-cell signalling, inflammation, cancer and angiogenesis [Arpicco et al., 2014; Trigkilidas & Anand, 2013]. GAGs have a variety of uses in the nutraceutical, pharmaceutical, and cosmetic industries due to their intriguing biological properties.

GAGs are currently extracted from discards of porcine/cattle slaughter houses and also from cartilages of whale and ray. GAGs from porcine/cattle are questionable due to risks of inter-species infections. Since whale and ray are potentially endangered, fish wastes are viable and safer sources of GAGs production [Menon & Lele, 2015; Geetha et al., 2022].

GAGs isolated from *Apostichopus japonicus* has structural similarity with chondroitin sulphate (→4GlcA (Fuc2S,4Sα1→3) β1→3GalNAc4S6Sβ1→), it has shown to have the ability to phosphorylate various signalling molecules such as IRS1, Akt and AMPK which are necessary to downstream processes of various essential pathways required for lipid metabolism including β-oxidation [Chen et al., 2019].

Studies showed ulvan, a type of sulphated polysaccharide, could alleviate HFD-induced weight gain and reduction in adipose tissue hypertrophy and triglyceride concentration in liver of experimental animals [Pung et al., 2022]. These sulphated polysaccharides are the bioactive polysaccharides which are well documented for their health beneficial effects due to their specialized sulfated modification which helps in the bioactivity of the polysaccharides [Telles et al., 2011].

Reports show that GAGs purified from an insect source i.e., *B. ignitus*, displayed anti-lipidemic effects in serum biochemical parameter levels and a change in the fatty acid composition of adipose tissues, showing an increased ratio of conjugated unsaturated fatty

acids in high fat diet fed rats and inferred that this compound might have potential for use as an anti-atherosclerotic agent [Ahn et al., 2017]. Nelson et al., (2015), studied diet induced obesity, and showed that Oralvisc^{TM} GAG mix, administered orally increased insulin sensitivity under both normal and high fat diets.

According to a report, GAGs isolated from the viscera of fish processing waste from *Pacu* and *Tilapia* showed anti-coagulant activity [Nogueira et al., 2019]. GAGs also have been isolated and identified from other marine sources such as sea cucumber (*Apostichopus japonicas*) which are proven to display immunostimulatory activity [Wang et al., 2017].

As explained above many studies reported that GAGs are important biomolecules with significant health benefits. However, the potential role of GAGs in ameliorating obesity induced atherosclerosis is not explored. In view of bioactive properties of GAGs, it is hypothesized that they might be beneficial to mitigate obesity related complications. Thus, the present study aimed to explore the impact of GAGs isolated from fish waste on obesity associated complications specifically atherosclerosis. The study also focused on understanding the underlying mechanisms by GAGs to alleviate the disease condition through *in-vitro* and *in-vivo* studies.

Hence, the present work aims at characterization of the isolated bioactive polysaccharide-GAGs from mackerel fish waste and its applications as a health beneficial molecule in high fat diet induced obese animal models in reducing obesity and also in reducing the obesity induced atherosclerosis, which is one of the complications caused through obesity.

The following objectives were envisaged in the proposed research work.

Objectives

i. To Isolate the Glycosaminoglycans from processed fish waste and evaluate its biological activity by *in-vitro* studies.

ii. To study the anti- atherogenic property of glycosaminoglycans in high fat induced obesity and identification of protein biomarkers.

iii. To understand the mechanisms involved in amelioration of anti-atherogenic properties linking to obesity.

iv. To formulate an anti-obesity food product and its storage stability studies.

The thesis is divided into six chapters. Chapter 1 deal with a General Introduction, Chapter 2, Material and Methods, Chapter 3, deals with Result and Discussion, Chapter 4 says about Summary and Conclusions. After chapter 4, a collective bibliography for all the chapters, list of publications and a list of posters presented are included.

Chapter 1: General Introduction

This chapter comprises a general introduction and review of literature for the fish catch and the waste generated during the processing globally and in the country, fish waste as a source of important recoverable biomolecules which includes bioactive polysaccharide-Glycosaminoglycans (GAGs), structure and functions of these GAGs will be explained for its role in reducing obesity, lipid metabolism and its related complication and our target obesity induced complication-Atherosclerosis. Further, literature evidences showing GAGs from various source as an important biomolecule in health and wellness will be further documented. Obesity, atherosclerosis, its patho-physiology, insulin resistance, adipose

tissue released adipokines and impacts of these cytokines on obesity and obesity induced atherosclerosis are discussed. Finally, the role of GAGs isolated from mackerel fish waste and their role in reducing obesity and its related complication atherosclerosis are discussed. At the end of this chapter, the scope of the present investigation is discussed.

Chapter 2: Materials and Methods

Chapter two includes Materials and Methods, extraction of GAGs from mackerel fish waste by chemical and enzymatic methods has been explained in a detail, the isolated GAGs is hereafter referred in the present study as extracted GAGs (e-GAGs), identification of isolated e-GAGs was done by agarose gel electrophoresis, its compositional analysis was analysed *in-vitro*, to further confirm the type of e-GAGs, it was digested enzymatically with specific and non-specific enzymes. Isolated e-GAGs partial characterisation was carried out by various analytical methods which includes FTIR, SEM. Structure elucidation of the isolated e-GAGs was done using ^{1}H NMR.

In-vitro anti-obesity property of e-GAGs was analysed by its property in inhibiting the lipase enzyme and as a bile acid binder to reduce the obese conditions. Further, lipid accumulation reducing property was checked in 3T3L1 preadipocytes, DNA protection, anti-coagulation property checked *in-vitro* in dose dependent manner.

In-vitro fermentability of e-GAGs isolated from fish waste was analysed with health beneficial gut microbes *Lactobacillus plantarum* (LP) and *Bifidobacterium bifidum* (BB), to produce health beneficial short chain fatty acids (SCFAs), which are beneficial in alleviating obesity and its related complications.

Animals and protocols for animal experiments, were carried out with C57BL/6 male mice models, which had IAEC clearance, two animal studies were carried out, one is diet induced

obesity model, and the other is obesity induced complication-Atherosclerosis (low dosage of STZ-60mg/kg body weight, i.p.), grouping and induction of obesity through high fat diet (40% fat), dosage of diet feeding with or without e-GAGs following standard AIN-93 diet formulation, the protocols have been further detailed.

In the animal experiments, anti-oxidant enzyme activities mainly SOD, GPx, catalase and lipid peroxidation were performed. Total lipid extracted from liver tissue and fecal matter, followed by its fatty acid composition analysis. Fecal bile acid estimation, plasma cholesterol, triglycerides, LDL-C, HDL-C and other liver markers like SGOT, SGPT, LDH *etc.,* were estimated, measurement of plasma insulin, inflammatory proteins were analysed by ELISA methods, tissue histopathology staining methods are described. Methods for estimation of protein content, western blot analysis, cDNA synthesis and RT-PCR analysis are presented in detail. Development and formulation of gummies with added e-GAGs were summarised for the textural, sensorial and storage stability studies.

Finally, statistical analysis was employed, all data were expressed as mean ± SEM and data were evaluated by GraphPad Prism software version 5.0 (GraphPad Prism Inc., San Diego, California) and the differences between the average values were assessed using Analysis of Variance (ANOVA) followed by Duncan's multiple range test (DMRT) presentation of results and statistical significances are given and the statistical significance was considered at $p < 0.05$.

Chapter 3

Result and Discussion: This chapter covers all the objectives in the subsections and contain results and discussion along with of the experiments carried out. Obtained results has been discussed along with references has been detailed in the thesis. Here only, the obtained results of each individual objective have been addressed under the following sections.

Chapter 3.1: Isolation of glycosaminoglycans (GAGs) from mackerel fish waste, its compositional analysis and partial characterisation

Mackerel fish waste which was procured from the local fish market in Mysuru, India, was used for the isolation of glycosaminoglycans (GAGs). Fish waste was subjected to its compositional analysis like moisture (76%), fat (7%), protein (16%) and carbohydrate (1%). Our study compound of interest hereafter referred as extracted glycosaminoglycans (e-GAGs), the yield of the e-GAGs from fish waste was found to be (963.5mg/g). The e-GAGs obtained were estimated for the compositional analysis like moisture(12.48%), total sugars (839.4mg/g), uronic acid (783.62mg/g), protein (33.46mg/g) and sulfate (210mg/g), this confirmed that the isolated compound was GAGs, it showed the typical composition for glycosaminoglycans, further it was also analysed for any contamination with macromolecules like protein, which was very negligible and nucleic acids was completely absent. So, it was confirmed that the extracted molecule was bioactive GAGs which possess biological activity. To further confirm the type of e-GAGs, it was digested enzymatically with specific and non-specific enzymes, results showed that the major e-GAGs was chondroitin sulphate (87.8%). Functional group identification in the e-GAGs was done by FTIR method, and it showed typical chromatogram which was compared with the standards, SEM analysis showed irregular cloudy structures. Further, structure elucidation was of the e-GAGS was done using ^{1}H NMR, the structure was that of Chondroitin Sulphate A (CS-A) showing alternating 3-linked N-acetyl α-galactosamine (GalNAc) units and 4-linked β-D-glucuronic acid (GlcA). The obtained results were encouraging to know that the e-GAGs can be used as a potent biologically active molecule for the further study. With this confirmation studies of the e-GAGs, *in-vitro* and *in-vivo* biological activity for obesity and its related complications in high fat fed mice models were further studied to understand its health beneficial effects in the further objectives.

Chapter 3.2: *In-vitro* biological activity of the extracted glycosaminoglycans (e-GAGs) isolated from mackerel fish waste as an anti-obesity potential

In this chapter, *in-vitro* anti-obesity activity was studied for pancreatic lipase enzyme inhibition, which was verified with the in-silico docking studies, DNA protection effects of e-GAGs was studied, e-GAGs was also studied as a bile acid binder, bile acids are important for the emulsification of fat and fat accumulation, blood clotting assays was analysed to check the anti-coagulation property of e-GAGs to reduce the other co-morbidities of obesity. 3T3L1 preadipocytes were used to study the effect of e-GAGs in reducing the lipid accumulation and also in reducing the differentiation of the adipocytes. *In-vitro* fermentation of e-GAGs was carried out to check its prebiotic property on the gut microbiota as a beneficial component for the growth of beneficial microbes and to estimate the secondary metabolites like short chain fatty acids (SCFAs) produced on fermentation, which are known for their health beneficial molecules in obesity conditions.

In-vitro blood coagulation showed various concentrations of e-GAGs (25,50,75,100mg/mL) reduced the blood from coagulating in dose dependent manner. The 3D structure of the enzyme lipase (1N8S) was retrieved from PDB and molecular interaction of the ligand and macromolecules were confirmed. Heparan sulphate had highest binding energy (-5.8 kcal Mol^{-1}) indicating its high affinity towards lipase enzyme while chondroitin sulphate and hyaluronic acid was observed to be -5.6 kcal Mol^{-1} and -5.0 kcal Mol^{-1} of binding energy, respectively. These energies are enough for GAGs and enzyme interactions. Thus, e-GAGs may acquire the property of lipase enzyme inhibition and thereby reduce its participation in lipid metabolism.

In-vitro biological activity showed good lipase inhibition, e-GAG showed lipase inhibition at IC_{50} value of 2.6mg/mL, commercial orlistat (Orl) drug had an IC_{50} value of 0.029mg/mL ($p<0.01$), while the enzyme digested e-GAGs by hyaluronidase (HYL) and Chondroitinase

ABC (Ch ABC), did not show very good lipase inhibition property-24.5 & 22.3 mg/mL respectively, indicating e-GAGs in its native form can reduce lipid absorption through inhibiting the lipase enzyme activity.

Bile acid binding study for the e-GAGs, was conducted with the positive control (cholestyramine) and test samples (e-GAGs), which were incubated with known concentration of bile acids mixture and analysed by HPLC. The results clearly indicated there is a clear reduction in the peak intensity of bile acid with increasing the concentration of e-GAGs of different doses. The HPLC peak for bile acid obtained from positive control and the e-GAGs chromatogram at different doses for bile acid binding showed the reduced peak intensity (mAU) with increasing concentration of e-GAGs. This indicates that e-GAGs has ability to bind bile acid and make it unavailable as a free bile acid for the fat emulsification.

3T3L1 preadipocytes cell lines showed reduction in lipid accumulation with e-GAGs treatment at various concentrations of 15, 30, 60 & 75µg/mL. Oil Red-O stain was used to analyze the lipid accumulation, after treatment with e-GAGs lipid accumulation was reduced in dose dependent manner and stain intensity was measured at 520nm.The result indicated that the e-GAGs inhibits accumulation of lipids within cells.

In-vitro fermentability of e-GAGs extracted from mackerel fish waste was analyzed with *Lactobacillus plantarum* (LP) and *Bifidobacterium bifidum* (BB), e-GAGs at 0.5 and 1% proved their prebiotic nature up to 48 hrs. The pH value decreased from 6.23 to 3.32, cell density increased from 1.70 to 2.32, viable cell count increased from 8 to 12 log CFU/mL, short chain fatty acids (SCFAs) production was ≈ 33, 31 and 36% for LP & ≈ 37, 29 and 34% for BB of acetic acid, propionic acid and butyric acid respectively.

This chapter concluded that the e-GAGs treatment is effective as an anti-obesity candidate which was substanti-ated with our generated data *in-vitro* and in-silico.

Chapter 3.3: Animal studies

Chapter 3.3.1: *In-vivo* studies for extracted glycosaminoglycans (e-GAGs) isolated from mackerel fish waste as an anti-obesity potential molecule in high fat fed animal models

In-vivo animal experiment was conducted in C57BL/6 male mice models and had IAEC clearance (#150/2019), these mice models are considered as an appropriate animal model for obesity, treatment of e-GAGs was given through diet along with high fat diet (40% fat). Five groups of animals were maintained **SFD** (Starch fed diet/Control), **HFD** (High fat diet-40% fat), **OFD** (Positive control- Orlistat fed diet along with high fat) & e-GAGs treatment group **GFD1 & GFD2** (380 & 760 mg/kg diet) respectively through diet along with high fat diet.

High fat feeding (40% fat) was used in the study diet. The results showed ameliorative effects of e-GAGs compared with the HFD group *via.,* reducing blood sugar ~1.28-fold, lipid profile, plasma insulin by ~3.5-fold, improved glucose tolerance, and homeostatic model assessment for insulin resistance (HOMA-IR, ~3.0-fold). Furthermore, elimination of bile acids (BAs) due to e-GAGs- BAs binding properties resulted in removal of elevated fecal triglyceride and cholesterol suggesting its lipid lowering activity. Regulation of various proteins linked to carbohydrate and lipid metabolism including fatty acid synthase (FAS), low density lipoproteins receptor (LDL-R), 7α-hydroxylase, glucose transporter-4 (GLUT4) and Peroxisome proliferator- activated receptor gamma (PPAR-γ**)** were significant ($p<0.05$) with e-GAGs treatment when compared to HFD group.

In the glucose tolerance test, the value of AUC of HFD fed group was higher when compared with SFD group, in the treated groups blood glucose tuned to their basal level drastically when compared with HFD group and were highly significant ($p<0.05$). Furthermore, increased insulin levels and HOMA-IR showed the clear indication of insulin resistance in the HFD group and were significantly ameliorated in the treatment groups in dose dependent manner.

To further understand e-GAGs effects on the insulin sensitivity at cellular level it was found that muscle GLUT4 expression was enhanced with increased e-GAGs dosage. Furthermore, increased expression of LDL-R and 7α-hydroxylase (CYP7A1) were observed in liver of e-GAGs treated groups. The removal of bile acid by e-GAGs with its bile acid binding properties lowers the bile acid in blood thus enhances insulin sensitivity, achieving normal blood glucose and accordingly reduces the secondary complications of obesity. Furthermore, PPAR-γ is a transcription factor which play major role in carbohydrate and lipid metabolism. e-GAGs showed increased expression of PPAR-γ, expressions of LDL-R and CYP7A1 were tuned to normal level, which helps in reducing the conversion of cholesterol to bile acid, which further helps in reducing the fat emulsification. Overall, e-GAG has the ability to disturb enterohepatic circulation *via.*, its bile acid sequestering property. Furthermore, down regulation of fatty acid synthase (FAS) expression was observed in the liver of e-GAGs treated groups.

Analysis of fecal matter showed the elimination of TG and cholesterol in the treated group. Treatment with e-GAGs showed dose dependent removal of fat and cholesterol. In addition, fecal bile acid elimination was higher in the e-GAGs treated groups. Histopathology of liver tissue shower the enlarged hepatocytes in the HFD group, but was normalised with the e-GAGs treatment.

Plasma biomarkers like SGOT, SGPT, ALP, creatinine & LDH were reduced in the treatment groups in dose dependent manner compared to HFD group. Fatty acid composition showed HFD group liver had highest saturated fatty acids (54.25%) and was ameliorated to 50.25 & 48.35% with the e-GAGs treated GFD1 & GFD2 respectively. Fecal fatty acid composition showed the higher ($p<0.05$) elimination of SFAs in the treatment groups. Tissue histopathology of spleen, kidney & pancreas showed highest damage in the high fat fed group and normalized with the e-GAGs treatment, damage score in high fat fed group was 9.36 (spleen), 6.73 (kidney) & 8.65 (pancreas), the scores reduced in the treatment groups to 5.12, 2.12 & 4.12 respectively. The intestinal disaccharidase enzymes activity reduced with the e-GAGs treatment, maltase activity reduced by 1.94 &2.04-folds, sucrose activity reduced by 1.88 &2.86-fold and lactase activity reduced by 1.41 &1.59-folds with the treatment groups ($p<0.05$) compared to HFD group. Gene expression studies in liver and adipose tissue showed e-GAGs treatment groups had down-regulated the lipid metabolism & inflammatory genes, liver tissue reduced expressions of SREBP-2 (2.82 & 3.51-fold), ACC1(2.5 &3.44-folds), (5.15 &5.31-folds) and TNF-α (2.34 & 2.98-folds). Adipose tissue showed the reduced expressions of SREBP1c (1.46 &1.76-folds) &ACC1 (1.26 &1.65-folds), TNF-α (3.06 &5.73-folds) and IL-6 (2.46 &3.18-folds) in GFD1&2 groups respectively compared to HFD group. e-GAGs could be beneficial in modulating obesity *via.,* molecular gene expressions and modulation of fatty acid composition.

e-GAGs treated animals ameliorated gut microbiome and anti-oxidant enzyme activity. e-GAGs fed group significantly decreased the lipid peroxidation,and ameliorated the activities of catalase, SOD and GPx . The treated mice also showed the amelioration of various parameters such as anti-inflammatory (CRP & TNF-α). Cecum microbial analysis showed health promoting effects of e-GAGs in reducing ($p < 0.05$) the obesity ratio of *Firmicutes* to *Bacteroidetes* (F/B) within the range (5.32 & 5.26) when compared with HFD (6.23). Hence,

e-GAGs can be a potential molecule for obesity by restoring the redox status under oxidative stress as well as gut microbes that generate SCFAs like acetate, propionate and butyrate which are known to have health beneficial effects.

Plasma inflammatory markers like TNF-α and CRP were high in HFD group, treatment with e-GAGs showed amelioration in dose dependent manner in GFD1& 2. TNF-α levels ameliorated by 0.58 & 0.54-fold and CRP levels by 0.64 & 0.61-fold in GFD1& 2 treated groups respectively, compared to HFD group

Plasma MDA (TBA reactant) level was increased in HFD group by 1.63-fold when compared to SFD and reduced with GFD1 and GFD2 by 1.09 and 1.22-fold, respectively compared to HFD and this indicates that e-GAGs are effectively involved in reducing lipid peroxidation. Anti-oxidant (AOX) enzymes like catalase, SOD and GPx activities were analysed in plasma, liver, kidney and heart.

Liver anti-oxidant results showed that level of MDA (TBA reactant) was increased 2.1-fold in HFD group upon treatment (GFD1 & GFD2) reduced by 1.2 and 1.43-fold, respectively. Catalase, SOD & GPx activity in HFD were found 1.8, 2.0 &1.9 with treatment 1.5 & 1.6-fold, 1.11 & 1.21-fold and 1.16 & 1.54-fold increased with GFD1 and GFD2 group respectively in dose dependent manner. The level of MDA in Heart (TBA reactant) was increased 1.25-fold in HFD group and reduced in GFD1 and GFD2 by 1.1 and 1.2-fold respectively. Catalase, SOD and GPx ameliorated in GFD1 and GFD2 group respectively in dose dependent manner

Effect of e-GAGs treatment on abdominal fat accumulation was assessed, the amount of visceral (abdominal) fat and the size of adipocytes by H&E staining was visualized. Histological staining by H&E of the adipose tissue in GFD1 &GFD2 groups showed significant decrease in the adipocyte size when compared to HFD. Adipose index was

increased 1.4-fold in HFD when compared to SFD and was reduced 1.23 &1.15-fold with GFD1 & 2 treated groups, respectively

Cecum collected from the sacrificed animals analysed for the *Firmicutes* to *Bacteroidetes* (F/B) ratio, an important indicator of obesity. HFD mice showed 6.23, the values tuned to normal (4.53) after treatment with e-GAGs (5.3 &5.2) are significant and in dose dependent manner. Microbial changes were analysed for different microbes specific for obesity conditions, health promoting bacteria like LAB & *bacteriodes* were ameliorated with the e-GAGs treatment, LAB reduced by 1.05-fold in HFD when compared to SFD, and increased 1.03 & 1.04-fold in treated groups, *bacteriodes* reduced 1.37-fold in HFD group. However, treatment with e-GAGs of both the groups were increased by1.0-fold. Opportunistic bacteria count was reduced with treatment groups by ≈1.0-fold in the GFD1&GFD2 groups. The *in-vivo* study with high fat fed mice models showed the amelioration of various biomarkers of inflammations, anti-oxidant status and adiposity after treatment with e-GAGs from fish waste. Further, support the growth of microbes that augment the *Firmicutes/Bacteroidetes* (F/B) ratio and condemned obesity development.

Chapter 3.3.2: *In-vivo* studies for extracted glycosaminoglycans (e-GAGs) isolated from mackerel fish waste as an anti-obesity potential molecule in high fat fed obesity induced complication: Atherosclerosis

In-vivo studies was conducted in C57BL/6 male mice models (IAEC# 224/2021) with high fat diet (40% fat) and low dose STZ (60mg/kg body weight i.p) for inducing the obesity and its related complications and e-GAGs treatment was through diet i.e. (380 & 760 mg/kg diet) along with high fat diet. Sacrificed animal tissues were used to analyse for anti-oxidant potential and plasma estimated for biochemical parameters by commercial kits and ELISA methods. Changes in molecular gene expressions was studied in liver and heart tissue.

In-vivo animal studies showed, treated groups had ameliorated plasma lipid profile and insulin levels by ~1.3 & 2.5-fold respectively. Liver and heart tissue anti-oxidant enzymes ameliorated with the treatment groups. Inflammatory markers like CRP and TNF-α with e-GAGs treatment significantly decreased by 3.2 & 3-folds respectively. HbA1c which is a marker for the chronic elevated blood glucose showed that the STZ induced high fat fed animals' values was higher than 12.23%, but with the e-GAGs treatment it reduced to 5.12%, and was tuned to normal, thereby reducing the obesity related complications.

Histopathology for the liver, kidney and heart tissue showed the reduced disintegration of tissue morphology and reduced lipid accumulation in the liver tissue with e-GAGs treatment when compared with the STZ high fat fed group.

To gain insight into the pathophysiology of obesity was done by comparing the pattern of gene expression. Down-regulation and up-regulation in the expression of several genes in heart and liver tissues was observed. Their expressions may be involved in the etiopathogenesis of obesity induced atherosclerosis.

In the liver tissue, lipid metabolism related genetic expression and mRNA levels of ACC1, SREBP1c and SREBP2 were down-regulated in e-GAGs treated groups by 1.46, 1.29 & 2.52-folds in GFD1-D; 1.58, 1.43 & 3.36--folds in GFD2-D respectively. Whereas, CYP7A1 gene expression was up-regulated by 2.73 & 3.22-folds in GFD1-D &GFD2-D groups respectively compared to HFD group. Similarly, inflammatory related gene expression of NF-κB and TNF-α were down-regulated in e-GAGs treated groups by 3.03 & 3.39-fold for NF-κB and 2.01 & 2.87-fold for TNF-α in GFD1-D & GFD2-D groups respectively compared to HFD.

In the heart tissue, genetic expression of inflammatory related genes which were related to obesity induced atherosclerosis disease progression were studied. mRNA levels of cd36, ICAM, VEGF and VCAM expressions were down-regulated by dose dependent manner in

e-GAGs treated groups by 1.62, 1.14, 3.57& 1.86--fold in GFD1-D; 1.73, 1.28, 4.17 & 2.16 in GFD2-D groups respectively compared to HFD. Similarly, inflammatory related gene expression of NF-κB and IL-6 were down-regulated in e-GAGs treated groups by 1.08 & 1.26-fold for NF-κB and 2.01 & 2.63-fold for IL-6 in GFD1-D & GFD2-D groups respectively compared to HFD. Hence, mRNA expressions indicated that the results were specific and significant for down-regulation of fatty acid and cholesterol biosynthesis and are responsible for the obesity induced/linked atherosclerosis and intervention of e-GAGs tuning the lipid homeostasis that implicate the athero-protective effects.

Gene expression studies of liver and heart tissue with e-GAGs treatment showed the reduced expression of specific proteins related to obesity induced atherosclerosis, which was compared with the HFD fed animals. This chapter confirmed that the extracted e-GAGs molecule from fish waste acts as an anti-obesity agent and also reduced the obesity complications including atherosclerosis. Therefore, has both industrial and pharmaceutical applications.

Chapter 3.4: Development of gelatin based gummies with added extracted glycosaminoglycans (e-GAGs) as an anti-obesity product

e-GAGs was used as a nutraceutical in development of gelatin based gummies, the product was successfully formulated and supplemented with bioactive e-GAGs. Low calorie sugar substitute like maltitol and added e-GAGs at 1.5% was a suitable formulation, incorporation of e-GAGs into the gummies did not significantly impact the textural properties of the gummies and were comparable with control, therefore it can be labelled as a low calorie product with added nutraceutical. The impact of sugar replacement and e-GAGs addition were evaluated in terms of quality characteristics and nutraceutical retention of gelatin gummies. The two formulations of gummies were prepared, sugar (S) and no added sugar

(NAS); where sugar was replaced with maltitol, a low calorie sweetener. e-GAGs was supplemented at 0.5, 1.5 and 3% (*w/w*) in both. Storage studies (6 months, 25°C in RT) showed no significant changes in its textural, sensory properties and were microbiologically safe, the added e-GAGs were extractable up to 95%. Gummies with maltitol and e-GAGs at 1.5% was a suitable formulation as an anti-obesity product.

Overall, fish waste was successfully used to isolate health beneficial and bioactive molecule glycosaminoglycans, which otherwise is getting wasted, and also adding to the environment pollution in the marine and coastal regions, our study of extracting the biomolecule from fish waste also adds to the waste to wealth concept. Hence, we may conclude that the e-GAGs had beneficial effects in reducing the obesity and its related complication like atherosclerosis which was analysed through the *in-vitro* and *in-vivo* studies. This study also confirmed that the extracted GAGs molecule from fish waste has both industrial and pharmaceutical applications as an anti-obesity agent and also reducing the obesity complication including atherosclerosis.